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Review

tRNA-nucleotidyltransferases: Highly unusual RNA polymerases with vital functions

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ABSTRACT

tRNA-nucleotidyltransferases are fascinating and unusual RNA polymerases responsible for the synthesis of the nucleotide triplet CCA at the 3′-terminus of tRNAs. As this CCA end represents an essential functional element for aminoacylation and translation, these polymerases (CCA-adding enzymes) are of vital importance in all organisms. With a possible origin of ancient telomerase-like activity, the CCA-adding enzymes obviously emerged twice during evolution, leading to structurally different, but functionally identical enzymes. The evolution as well as the unique polymerization features of these interesting proteins will be discussed in this review.

1. Introduction

Life in all forms as we know it crucially depends on biological active macromolecules made up from individual building blocks, like nucleic acids, proteins, glycans and many others. For the synthesis of DNA and RNA, a huge set of different polymerase enzymes are identified in all organisms. Besides the classical polymerization enzymes for replication and transcription, several polymerases fulfill specific repair or maintenance functions on nucleic acids, ranging from translesion DNA polymerase (a classical DNA repair enzyme) to telomerase (chromosomal end maintenance), poly(A) polymerase (3′-end tailing of RNA) and others [1–5]. A specific role is carried out by ATP(CTP):tRNA nucleotidyltransferase – a polymerase that synthesizes the highly conserved sequence CCA to the 3′-end of tRNAs ("CCA-adding enzyme") [6,7]. This specific enzyme differs from the above mentioned polymerases in several ways. First, it incorporates only a highly restricted number of nucleotides in a tRNA primer and then stops polymerization at a high efficiency and accuracy. Second, it selects exclusively CTP and ATP for incorporation and discriminates strongly against the other two nucleotide triphosphates. Third, it does not require a nucleic acid template for directing order and nature of nucleotides to be inserted. Fourth, this polymerase is highly selective for tRNA-like structures as a polymerization substrate. Fifth, it fulfills both functions in maintenance/repair as well as de novo polymerization. Sixth, this type of enzyme emerged twice in evolution, leading to structurally different proteins with identical functions.

2. A classical view of a polymerase

In general, nucleic acid polymerization is composed of three individual phases. Initiation describes the touchdown of the polymerase on the template, the recognition of the synthesis starting point and the incorporation of the first nucleotide(s). The subsequent elongation comprises either a processive polymerization reaction, where one nucleotide after the other is incorporated by the polymerase without leaving the template. Alternatively, in a distributive elongation mode, the polymerase dissociates from and re-associates with the template, starting the synthesis anew. Termination is a (more or less efficiently) controlled stop of polymerization and defines therefore the length of the resulting product.

This view gives rise to three essential questions: (a) How is the template selected that is used to define nature and order of the building blocks to be added? (b) How does the polymerase discriminate the individual but very similar nucleotides? (c) When and how does the polymerase stop its action? Most polymerases use external signals like a primer on the template strand (DNA polymerases) or a promoter sequence (RNA polymerases) for finding their starting position. Alike, stop signals are used by RNA polymerases for ending the polymerization reaction like terminator structures in the transcript (prokaryotes) or phosphorylation signals on a specific polymerase domain (C-terminal domain; eukaryotic RNA polymerases). While eukaryotic termination of replication is still quite unclear, bacterial DNA polymerases are stopped by contra-helicases (Tus proteins) that bind to Ter sequences of the template and block the helicase activity of the replication machinery [8–10]. In the laboratory, however, a run-off reaction is used, where the polymerase is simply falling off at the end of the

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template (e.g. in vitro transcription, PCR). The selection of specific nucleotides to be incorporated in a sequence-dependent way is of course dictated by the nucleic acid template that the classical DNA as well as RNA polymerases depend on.

The chemical mechanism underlying the polymerization appears stunningly conserved in all polymerases across the three kingdoms of life. Two metal ions are coordinated by highly conserved carboxylates and fulfill specific roles in catalyzing the reaction. Metal ion A activates the 3′-hydroxyl group of the primer for a nucleophilic in-line attack on the alpha-phosphate of the incoming NTP, while metal ion B promotes the leaving of the pyrophosphate group that is released during this reaction [11]. Proceeding to the next nucleotide to be incorporated, DNA as well as RNA polymerases synthesize their nascent nucleic acid polymer in a 5′–3′ direction.

As the tRNA-nucleotidyltransferases carry an active site with similar carboxylate positions for binding two catalytically important metal ions, the CCA-addition is based on the same polymerization mechanism [12,13]. This is further supported by the fact that structural overlays of the catalytic core display a highly similar organization of this domain in all nucleotidyltransferases [14–16]. Hence, this motif can be used as a hallmark for nucleic acid polymerases [17]. However, that is about where things start to get different for the CCA-adding enzymes. Nucleotide selection, sequence specificity and polymerization start and stop signals differ dramatically from the above mentioned standard polymerases. The peculiar solutions for these problems found in the tRNA-nucleotidyltransferases make this class of enzymes so fascinating and worth-while to study.

3. Special case of the tRNA-CTP, ATP-nucleotidyltransferase

While the catalytic activity of CCA-adding enzymes is also based on the described two-metal-ion-mechanism, these enzymes show very unique features in several aspects. Instead of an external nucleic acid–based template, amino acid residues in the nucleotide binding pocket contribute to the high sequence specificity during synthesis [13,18]. Furthermore, CCA-adding enzymes obviously can count until three: after the addition of three nucleotides, the polymerization reaction is efficiently stopped, while other polymerases like T7 RNA polymerase or the Taq enzyme frequently add a non-templated extra nucleotide. These extra nucleotides hamper the synthesis of homogeneous T7 in vitro transcripts but are efficiently exploited for convenient cloning of PCR products [19]. The CCA-adding enzymes, however, add only under extreme artificial in vitro conditions more non-templated residues to the tRNA primer end [20–22]. Additionally, and most interestingly, the CCA-adding enzymes recognize if nucleotides are previously added to a tRNA primer and incorporate then only the missing ones, completing thereby the CCA triplet. A tRNA that carries already the first C residue of the CCA terminus is elongated only by one C and one A, while on a tRNA ending with CC, only the terminal A residue is added. This feature shows that CCA-adding enzymes are not only responsible for the de novo synthesis of CCA ends but have an important maintenance and repair function for tRNA ends. This stringent sequence and length control of the tRNA CCA end reflects the recognition requirements for aminoacylation and translation. Aminoacyl-tRNA synthetases as well as translation factors (EF-Tu) require a correct CCA terminus in order to accept a tRNA as a substrate [23]. Furthermore, positioning in the ribosome during translation [24] and even peptide release from the ribosome depend on an intact CCA end, which is critical for water coordination and efficient hydrolysis of the ester bound translation product [25,26]. These facts indicate that an accurate CCA end participates, beyond simple recognition and binding, as an integral part in several reaction mechanisms and is therefore of vital importance for the cell.

Surprisingly, these polymerases with such unusual features evolved twice in evolution, leading to classes 1 and 2 CCA-adding enzymes [27]. While class 1 is exclusively found in archaea, class 2 tRNA-nucleotidyltransferases are present in eukaryotes and bacteria, where they fulfill identical functions. Although both classes share an overall structural organization, the individual domains vary dramatically and have different mechanistic solutions for the above mentioned features (Fig. 1). Class 1 enzymes have a tRNA-binding body domain consisting of a beta sheet with flanking alpha helices. Head and neck domains form the active site and are also composed of alpha-helical and beta-sheet elements [18]. In class 2 enzymes, however, only the head domain carries a beta sheet and forms the nucleotidyltransferase core, while neck, body and tail consist exclusively of alpha helices, giving the enzyme a hook- or seahorse-like overall structure [12,13].

One of the most fascinating aspects of both classes of tRNA-nucleotidyltransferases is the fact that CCA-addition does not require an external nucleic acid as a template – somehow these enzymes "know" when to incorporate which nucleotide. Crystal structures of both classes 1 and 2 enzymes revealed a set of highly conserved amino acid residues located in the single nucleotide binding pocket that interact with the incoming nucleotide by forming Watson/Crick-like hydrogen bonds [13,18,28,29]. However, the binding pocket of class 1 enzymes recognizes the bases only with a single highly conserved arginine residue and is therefore rather unspecific, tolerating in principle every type of nucleotide [18]. Specificity comes from the tRNA 3′-end that also interacts with the nucleotide to be incorporated. Here, the backbone phosphates interact with the bound CTP or ATP and additionally help to position the templating arginine in the correct orientation (Fig. 2) [30]. Hence, class 1 enzymes recognize and select the correct nucleotides not as pure protein-based enzymes, but as ribonucleoproteins, where the tRNA part is not just a substrate molecule (primer), but is an active part of the nucleotide binding pocket. Class 2 enzymes, on the other hand, select the nucleotides to be incorporated by a true amino acid template that consists of the three highly conserved residues glutamic acid, aspartic acid and arginine (EDxxR). The arginine residue forms hydrogen bonds with ATP (1 bond) and CTP (2 bonds), assisted by aspartate that contributes one hydrogen bond (Fig. 2) [13]. The importance of this protein-based template was demonstrated by replacing these residues by amino acid side chains with reversed hydrogen bond donors and acceptors. This led to a binding pocket that preferred UTP and GTP over CTP and ATP, showing an elegant reprogramming of the polymerization reaction [31]. However, only single U and G residues were incorporated, but not a complete UUG terminus. This indicates that other features aside the amino acid template participate in determining the specificity of the nucleotide addition. While a replacement of the arginine residue by alanine clearly abolished nucleotide selectivity of the binding pocket and let to a dramatically increased rate of misincorporations, the mutant enzyme still synthesized a considerable amount of correct CCA-termini [32]. Again, the tRNA primer plays an important role, however, it is not participating in selecting the correct nucleotides to interact with the binding pocket. Instead, it seems that the incorporated C residue at the 3′-end of the tRNA primer is recognized by a second set of amino acids, leading to a specific orientation of the 3′-hydroxyl group of its ribose. This positioning is required for a nucleophilic attack of the 3′-OH at the alpha-phosphate of the bound (correct or incorrect) NTP [33]. However, if a wrong nucleotide was already incorporated and is now located at the 3′-end of the tRNA primer, this 3′-end cannot be positioned correctly, and no further nucleotide is added. Using such a 3′-end positioning mechanism, class 2 CCA-adding enzymes elongate only tRNAs with correctly added nucleotides. This selective polymerization represents an efficient back-up mechanism for CCA-addition

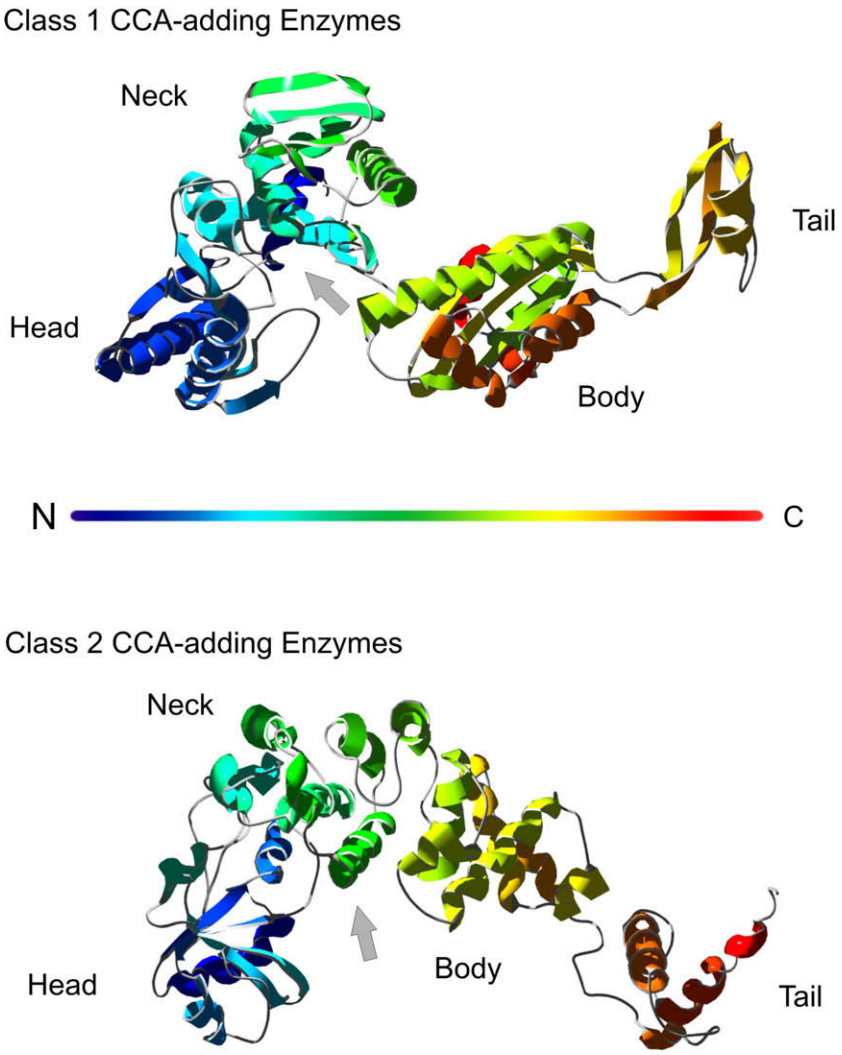

Fig. 1. Structural organization of classes 1 and 2 CCA-adding enzymes. While both enzyme versions have a hook-like shape of similar size, the allocation of secondary structure elements in neck, body and tail domains are quite different. In class 1 enzymes, these regions contain alpha-helical as well as beta-sheet elements. Class 2, on the other hand, has exclusively alpha-helical structures in these domains. The catalytic cores, located in head and neck domains of both enzyme versions, are indicated by the grey arrows. The rainbow color bar represents the consecutive protein regions from N- (blue) to C-terminus (red). The structures are extracted from the corresponding pdb file deposits of the protein data bank [13,30].

even in the presence of an actually deleterious mutation in the amino acid template [32]. Hence, while in class 1 enzymes nucleotide discrimination is primarily based on NTP binding (to enzyme and tRNA primer), class 2 enzymes select the nucleotides to be incorporated by binding (amino acid template) and catalysis (primer positioning). As a common feature for both enzyme classes, stacking interactions of the primer and the bound nucleotides in addition to the template-mediated hydrogen bonds further contribute to the observed high nucleotide selectivity during CCA-addition. This collaboration of protein and tRNA primer also explains how these enzymes recognize and complete partial CCA ends by the addition of the missing residues instead of synthesizing blindly complete CCA triplets at all times [28].

The existence of single nucleotide binding pockets that can be adjusted to accommodate either CTP or ATP requires a certain flexibility in both enzyme classes. For class 2 enzymes, such a conformational shift for switching from CTP to ATP incorporation is supported by the observation that a co-crystal consisting of a tRNA nucleotidyltransferase with bound tRNA primer (ending with CC-) readily dissolved upon soaking with ATP [33]. A possible hinge or cantilever element required for this readjustment of the binding pocket from C- to A-specificity was recently identified as a highly flexible element in class 2 CCA-adding enzymes, where manipulation or deletion of this region dramatically interfered with ATP incorporation [34,35] (Hoffmeier, unpublished). However, it is still unclear what represents the mechanical trigger to switch towards ATP recognition after the addition of two C residues.

The architecture of the binding pocket probably contributes also to the restricted number of nucleotides to be incorporated. Under standard conditions, addition of extra nucleotides is almost never observed. It is very likely that the binding pocket and the catalytic core of the enzymes cannot accommodate more than the three added residues at the tRNA end, forcing the enzyme to stop polymerization immediately. However, with the poly(A) polymerases in archaea/eukaryotes (class 1) and bacteria (class 2), both types of CCA-adding enzymes have close relatives without such a length restriction [27]. Interestingly, the overall organization of these poly(A) polymerases strongly resembles those of the tRNA-nucleotidyltransferases. Therefore, it is still a great mystery why length restriction occurs only in the CCA-adding enzymes but not in poly(A) polymerases. For class 2 enzymes, this restriction is obviously not mediated by the N-terminally located catalytic core

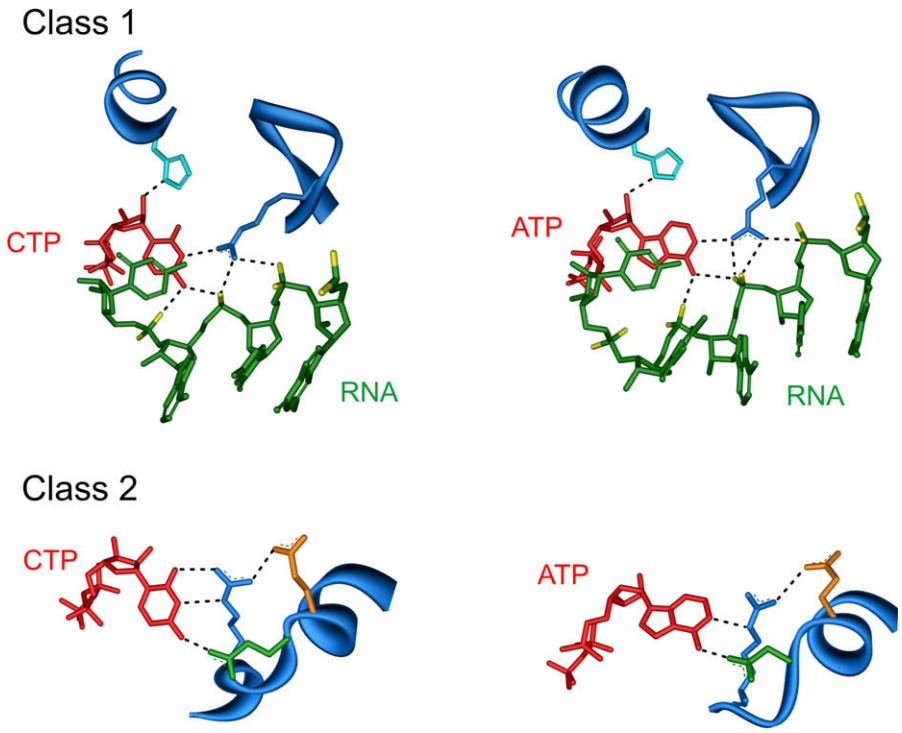

Fig. 2. Template regions of the nucleotide binding pockets in classes 1 and 2 enzymes. In class 1 enzymes, a histidine residue (cyan) interacts with the ribose moiety of the incoming nucleotide (red), while a highly conserved arginine (blue) recognizes the base. Nucleotide specificity comes from further interactions of the CTP or ATP with the phosphates (green and yellow) of the RNA primer backbone (green). In contrast, in the nucleotide binding pocket of class 2 enzymes, the nucleotides to be incorporated are recognized exclusively by a set of amino acid residues. An arginine (blue) is correctly positioned by the interaction with a glutamate (orange) and forms hydrogen bonds to the bound bases (red), assisted by an aspartate (green) that contributes a further H-bond. However, the positioning of the primer 3′-OH also contributes to the specificity of CCA-addition, as discussed in the text (not shown). As in Fig. 1, the partial structures are extracted from pdb entries of classes 1 and 2 CCA-adding enzymes [13,30].

alone, but by the less conserved C-terminal region. Replacing this protein part by the corresponding region of a class 2 poly(A) polymerase resulted in a chimeric enzyme (N-terminus and catalytic core: CCA-adding enzyme, C-terminus: poly(A) polymerase) that synthesizes multiple rounds of CCA-addition on a tRNA primer [36]. Consequently, the C-terminus must carry elements that are required for the restricted incorporation of three nucleotides.

Although the C-terminal region of class 2 enzymes is less conserved compared to the N-terminal part including the catalytic core and the nucleotide binding site, additional functions of this region could be identified in some enzymes. As an example, the *Escherichia coli* enzyme carries a C-terminal HD domain that represents a typical motif of metal-dependent phosphohydrolases [37]. Accordingly, this enzyme shows an efficient phosphatase activity removing 2′-3′-cyclic phosphate groups of tRNA transcripts [32]. This additional activity indicates that CCA-adding enzymes with an HD domain have a further function besides CCA-addition. One of the most prominent damages occurring on tRNAs is spontaneous hydrolytic cleavage. If parts of the CCA terminus are lost due to such a damaging reaction, the tRNA carries a partial CCA end with a 2′-3′-cyclic phosphate group that blocks the 3′-hydroxyl for immediate nucleotide addition by the CCA-adding enzyme. The phosphatase activity, however, can remove the cyclic phosphate and converts thereby the blocked 3′-end into a standard primer 3′-end, representing the ideal substrate for nucleotide addition and, consequently, repair of the CCA end. Thus, these associated activities of the CCA-adding enzyme allow a rapid restoration of damaged tRNA molecules catalyzed by one single protein [32]. Other class 2 enzymes, however, do not show such an additional activity and catalyze therefore predominantly the de novo synthesis of CCA ends. Nevertheless, it is possible that in these cases separate phosphatases are involved and participate in this tRNA repair.

4. Variations on the theme: CC- and A-adding enzymes

While CCA-adding enzymes represent universal and essential enzymes in tRNA maturation, it was a great surprise when tRNA-nucleotidyltransferases with partial activities were identified in several bacterial species. In these organisms, two class 2 enzymes were identified that collaborate in CCA-addition [34,38–40]. One enzyme adds exclusively the two C residues but fails to incorporate the terminal A. The second enzyme, however, has a complementing partial activity and completes the CCA end by adding the missing A residue. Interestingly, these CC- and A-adding enzymes show a high similarity to the standard CCA-adding enzymes at the sequence and structural level and cannot be easily distinguished from their evolutionary kinship. However, a recent analysis revealed that in the CC-adding enzymes, the above mentioned flexible hinge or cantilever element was deleted, inhibiting the enzymatic rearrangement required to switch from CTP to ATP binding and forcing these enzymes in a "CTP-only" configuration [34]. An artificial insertion of a corresponding hinge region restored the complete CCA-adding activity in such a CC-adding enzyme in vitro. These results support the idea that CCA-adding enzymes represent the ancestral enzyme form that evolved into CC-adding enzymes by the occurrence of this deletion. The evolutionary origin of the A-adding enzymes, on the other hand, is not clarified yet. In a phylogenetic analysis, these enzymes cluster in a discrete branch of the resulting tree and could represent either the original ancestral or a derived state of class 2 tRNA-nucleotidyltransferases [34].

The interesting coincidence that these enzymes with partial activities are predominantly found in extremophilic bacteria (*Bacillus halodurans*, *Deinococcus radiodurans*, *Aquifex aeolicus*, *Thermus thermophilus*, *Thermoanaerobacter tengcongensis*) might indicate that these extreme living conditions promote the occurrence of separated nucleotide adding activities. The possible scenario that the ancestral state of these bacteria might reflect that enzymes with partial activities represent the progenitors of modern CCA-adding enzymes is – at least for the CC-adding enzymes – unlikely, because this would require a position- and sequence-specific insertion of the cantilever element during evolution. A scenario based on an accidental deletion of a functional element (as reported for other proteins) is therefore much more likely [34]. Furthermore, in archaeal organisms thriving under similar extreme conditions, only single (class 1) enzymes with full CCA-adding activity are found. Hence, CC- and A- adding enzymes seem to be specific variants of class 2 enzymes found exclusively in bacteria. Why similar restricted activities are obviously absent in class 1 enzymes is currently not known.

5. Alternative substrates for the CCA-adding enzymes

In the recent years, an increasing number of reports appeared discussing alternative substrate RNAs for CCA-adding enzymes. Aside of implications concerning the co-evolution of this type of enzyme and its substrate(s), these cases are of interest in order to understand the enzymes substrate requirements. It seems that a rather conserved hairpin-like structure is required, mimicking the upper part of a tRNA molecule. One of the first examples for CCA-termini at non-tRNA transcripts came from Williams and Mulligan, who described massive CCA-addition on mitochondrial mRNA transcripts *rps12*, *cox2* and *atp9* in maize, probably catalyzed by the mitochondrial CCA-adding enzyme [41]. The function of these non-encoded posttranscriptional CCA-additions is unclear. However, as the CCA triplet was frequently found on truncated 3′-termini of these transcripts, a contribution of the CCA end to mRNA degradation was discussed. A second case of CCA-addition to transcripts other than tRNAs was observed in the human nucleus, where 65% of the spliceosomal U2 snRNA carry a CCA end [42]. Here, at least the terminal A residue is added post-transcriptionally. Further examples for non-encoded addition of complete or partial CCA-termini were found in higher plant chloroplasts [43], in *Arabidopsis* mRNAs [44] and at the 3′-end of tobacco mosaic virus RNA [45].

While the number of such CCA-additions to non-tRNA substrates is increasing, the biological relevance of these unusual nucleotide incorporations is still unclear. It is possible that these cases are just unspecific side reactions representing the substrate requirements of CCA-adding enzymes, as most of the transcripts carry a stem-loop element at the 3′-end, resembling the upper half of a tRNA. Accordingly, a similar single RNA minihelix is an efficient substrate for CCA-addition in vitro [32,46]. However, some of the maize mitochondrial mRNAs carry CCA ends at positions that do not fold into a stem-loop structure. Hence, a tRNA-like structure is not required in all cases. This is supported by the observation that at least the terminal A-addition – catalyzed by the *E. coli* enzyme – can occur on single stranded RNA molecules ending with CC [32].

6. Evolutionary considerations

The fact that a stem-loop structure at the 3′-end of a transcript is readily accepted as a substrate for CCA-addition greatly supports a theory about the evolutionary origin of the CCA-termini and the upper part of tRNAs. In their "Genomic Tag" hypothesis, Maizels and Weiner elegantly discuss that in the RNA world, such a CCA terminus might have been a tag sequence required as a start site for the replication of individual RNA molecules [47,48]. Accordingly, an ancient CCA-adding activity (possibly RNA-based at that time) represented the first telomerase, ensuring that RNA replication started at defined positions without loosing sequence information at the 3′-end in each round of replication. Hence, a CCA-tag would have been a label for RNA molecules to be replicated. The beauty of this hypothesis is that it explains the evolutionary origin of both CCA-adding activities as well as tRNAs. Both obviously served as tools required for an efficient and accurate initiation of replication in early days of evolution. It is a fascinating aspect that these original ancient functions of both substrate (tag) and catalyst (RNA or protein- based telomerase) were then replaced by other, equally important functions in the modern protein world. However, it is not clear yet why the protein-based CCA-adding activity then evolved twice, leading to only distantly related CCA-adding enzymes of classes 1 and 2.

In-line with a presumptive ancient function as a telomerase, the CCA-adding enzymes show a surprising high fidelity. Although currently no actual error rates are published, CCA ends with misincorporated nucleotides are only rarely detected. Only under rather artificial in vitro conditions (in the presence of Mn^{2+} ions instead of Mg^{2+} or deviating NTP concentrations), incorporation of CCC as well as poly(C) tails could be observed [20–22]. While this extended synthesis of homopolymeric RNA tails supports a close evolutionary connection of CCA-adding enzymes and poly(A) polymerases [27], the high fidelity of CCA-addition under physiological conditions underlines the vital importance of this reaction. For proper initiation of polymerization, any misincorporation by the ancient telomerase probably interfered with successful replication of the corresponding RNA. Erroneous CCA-addition by the modern protein-based enzymes have similar detrimental effects. tRNAs with incorrect 3′-termini are either not aminoacylated or can promote frame shifting during protein synthesis, leading to non-functional translation products [23,49]. Hence, although the original function of CCA-addition obviously changed during evolution, the requirement for error-free synthesis of this sequence persisted and led to remarkable unique RNA polymerases that continue to fascinate us.

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